

## Detection of Extended-Spectrum $\beta$ -Lactamases in Clinical Isolates of *Pseudomonas aeruginosa*

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With the occurrence of extended-spectrum  $\beta$ -lactamases (ESBLs) in *Pseudomonas aeruginosa* being increasingly reported worldwide, there is a need for a reliable test to detect ESBLs in clinical isolates of *P. aeruginosa*. In our study, a total of 75 clinical isolates of *P. aeruginosa* were studied. Nitrocefin tests were performed to detect the  $\beta$ -lactamase enzyme; isoelectric focusing electrophoresis, PCR, and PCR product sequencing were designed to further characterize the contained ESBLs. Various ESBL-screening methods were designed to compare the reliabilities of detecting ESBLs in clinical isolates of *P. aeruginosa* whose  $\beta$ -lactamases were well characterized. Thirty-four of 36 multidrug-resistant *P. aeruginosa* clinical isolates were positive for ESBLs. *bla*<sub>VEB-3</sub> was the most prevalent ESBL gene in *P. aeruginosa* in our study. Among the total of 34 isolates that were considered ESBL producers, 20 strains were positive using conventional combined disk tests and 10 strains were positive using a conventional double-disk synergy test (DDST) with amoxicillin-clavulanate, expanded-spectrum cephalosporins, aztreonam, and cefepime. Modifications of the combined disk test and DDST, which consisted of shorter distances between disks (20 mm instead of 30 mm) and the use of three different plates that contained cloxacillin (200  $\mu$ g/ml) alone, Phe-Arg  $\beta$ -naphthylamide dihydrochloride (MC-207,110; 20  $\mu$ g/ml) alone, and both cloxacillin (200  $\mu$ g/ml) and MC-207,110 (20  $\mu$ g/ml) increased the sensitivity of the tests to 78.8%, 91.18%, 85.29%, and 97.06%.

*Pseudomonas aeruginosa* is a leading cause of nosocomial infections, including pneumonia, urinary tract infections, and bacteremia. The infections can be particularly severe in patients with impaired immune systems, such as neutropenic or cancer patients (14).

Extended-spectrum  $\beta$ -lactamases (ESBLs) are enzymes that mediate resistance to extended-spectrum cephalosporins (ESCs), such as cefotaxime (CTX), ceftriaxone, and ceftazidime (CAZ), and the monobactam aztreonam (ATM) (12). Such enzymes are most commonly found in *Klebsiella pneumoniae* and *Escherichia coli* and have been recently detected in *Pseudomonas aeruginosa* at low frequency (2, 10, 15, 14, 20). Various tests have been developed to detect ESBLs. Most of the methods aimed to detect ESBLs in klebsiellae and other *Enterobacteriaceae* with little or no chromosomal  $\beta$ -lactamase activity, e.g., *E. coli* and *Proteus mirabilis* (3, 4, 6, 8, 18). Detection of ESBLs in species with inducible chromosomal  $\beta$ -lactamases, such as *Enterobacter* spp., proved a difficult task. *P. aeruginosa* presents further difficulties, because it not only has an inducible AmpC enzyme but also has a much greater degree of impermeability than *Enterobacteriaceae*, as well as efflux-mediated resistance (12, 16, 19, 21).

Tzelepi et al. showed that the combination of double-disk synergy tests (DDSTs) that combine amoxicillin-clavulanic acid (AMC) with cefepime (FEP) could efficiently detect the ESBL producers among *Enterobacter* spp. (19). Poirel et al. showed that the use of a cloxacillin (CLO)-containing plate that inhibited cephalosporinase activity might enhance the ability of the DDST

to detect ESBLs in *Acinetobacter baumannii* in a routine laboratory (16), and Aubert et al. did the same for *P. aeruginosa* (1). The only interference to detecting ESBLs that was considered in all of these methods was from the AmpC enzyme.

Phe-Arg  $\beta$ -naphthylamide dihydrochloride (MC-207,110) is the first characterized inhibitor of multiple resistance-nodulation-cell division transporters from *P. aeruginosa*. MC-207,110 potentiated the activities of the antibiotics that were substrates for MexAB-OprM, which included fluoroquinolones, tetracyclines,  $\beta$ -lactams, and rifampin, but did not potentiate the activities of antibiotics that were not substrates for MexAB-OprM. MC-207,110 did not have significant activity against strains lacking known efflux pumps (13).

In this study, we attempted to determine the detection frequencies of ESBLs in consecutive samples of clinical isolates of multidrug-resistant *P. aeruginosa* with the various plate-screening methods mentioned above, as well as isoelectric focusing electrophoresis (IEF), PCR, and PCR product sequencing of  $\beta$ -lactamases. These screening methods included using cloxacillin alone to inhibit the activities of the AmpC enzyme, using MC-207,110 alone to inhibit the activities of efflux pumps, or using a combination of both cloxacillin and MC-207,110 for their inhibiting effects.

### MATERIALS AND METHODS

**Bacterial strains.** A total of 75 clinical isolates of *P. aeruginosa* were non-repetitively and consecutively obtained from clinical specimens in the Huashan Hospital, Shanghai, China, from July to December 2004. Species identifications were done by using the Vitek automated identification system (BioMérieux, Marcy l'Etoile, France) and were confirmed by the API-GN system (BioMérieux).

**Antimicrobial susceptibility testing and screening for ESBLs.** Routine antibiograms were determined by the disk diffusion method on Mueller-Hinton (MH) agar (Oxoid Ltd., Basingstoke, Hampshire, England). The antimicrobial agents were purchased from BioMérieux, Marcy l'Etoile, France. The results

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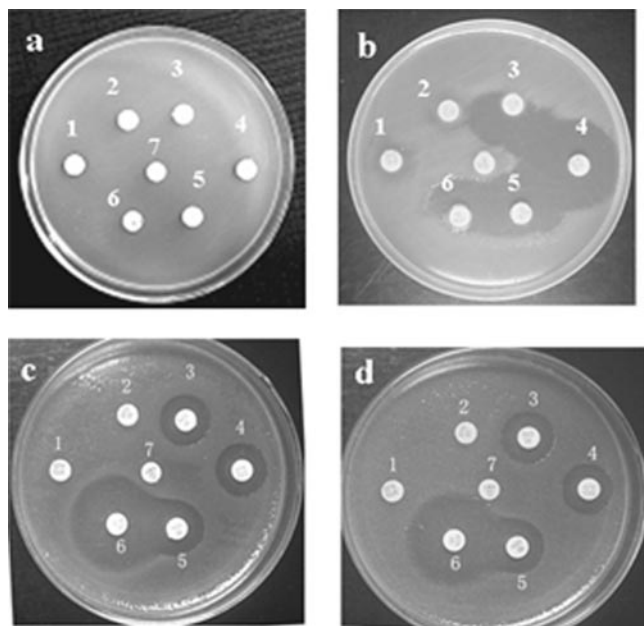


FIG. 1. Results of DDSTs and combined disk tests for detection of ESBLs in *P. aeruginosa* clinical isolates. The *P. aeruginosa* clinical isolate 970 (a and b), which is highly resistant to all antibiotics tested, was positive for ESBLs and the AmpC enzyme in IEF and PCR. The *P. aeruginosa* clinical isolate 843 (c and d), which is resistant to CAZ, CTX, FEP, AMC, CAZ/CLO, and CTX/CLO but susceptible to ATM, was negative for ESBLs and the AmpC enzyme in IEF and PCR. (a and c) DDSTs and combined disk tests performed on MH plates with disks of ESCs and ATM 20 mm from AMC. (b and d) DDSTs and combined disk tests performed on both cloxacillin (250 µg/ml)- and MC-207,110 (20 µg/ml)-containing plates with the disks of ESCs and ATM 20 mm from AMC. 1, cefotaxime-clavulanic acid; 2, cefotaxime; 3, ceftazidime; 4, ceftazidime-clavulanic acid; 5, cefepime; 6, aztreonam; 7, AMC.

were interpreted according to the CLSI (formerly NCCLS) standard. DDSTs were performed by placing disks of ceftazidime, cefotaxime, aztreonam, and cefepime (30 µg each) at a distance of 30 or 20 mm (center to center) from a disk containing AMC (amoxicillin, 20 µg, and clavulanic acid [CLA], 10 µg) (19). ESBL production was inferred when the cephalosporin zone was expanded by

the clavulanate. Combined disk tests (12), which were designed for detecting ESBLs in *Klebsiella pneumoniae* and *Escherichia coli*, were also performed by placing disks of ceftazidime, cefotaxime, ceftazidime-clavulanic acid, and cefotaxime-clavulanic acid on MH plates at a distance of 30 mm (center to center) from each other. ESBL production was inferred if the zones produced by the disks with clavulanate were  $\geq 5$  mm larger than those without inhibitor. In order to conveniently perform these two tests and observe the results, we integrated the DDSTs and combined disk tests into one plate by placing disks of ceftazidime, cefotaxime, aztreonam, cefepime (30 µg each), ceftazidime-clavulanic acid, and cefotaxime-clavulanic acid on MH plates as shown in Fig. 1. The distances of ceftazidime-clavulanic acid and cefotaxime-clavulanic acid from AMC, ceftazidime, and cefotaxime remained 30 mm, and the distances of ESCs, aztreonam, and cefepime from AMC were 20 mm. For ESCs, aztreonam, and cefepime, twice the measured half-diameter that was not affected by AMC was used as the zone diameter. The criteria for result interpretation were the same as those for the DDST and the combined disk test. DDSTs and combined disk tests were also performed on cloxacillin (250 µg/ml)-containing plates, MC-207,110 (20 µg/ml)-containing plates, and plates that contained both cloxacillin (250 µg/ml) and MC-207,110 (20 µg/ml).

**IEF of  $\beta$ -lactamases.** Nitrocefin tests were performed to detect the  $\beta$ -lactamase enzyme in clinical *P. aeruginosa* isolates according to the instructions in the kit. Development of a red color within 5 min in the area of the disk where the culture was applied implied  $\beta$ -lactamase production. *K. pneumoniae* ATCC 700603 and *E. coli* ATCC 25922 (obtained from Covance Central Laboratory Services Inc.) were used as positive and negative controls for  $\beta$ -lactamase, respectively. IEF was performed by electrophoresis of ultrasonic cell extracts of the  $\beta$ -lactamase producer on polyacrylamide gels containing ampholytes with pHs ranging from 3.0 to 9.0 (Amersham Pharmacia Biotech AB, Uppsala, Sweden).  $\beta$ -Lactamases were visualized with a 0.2-mg/ml nitrocefin solution (Oxoid Ltd., Basingstoke, England). The visualized  $\beta$ -lactamases were applied to one of three parallel focused gels without any treatment. At the same time, the visualized  $\beta$ -lactamases were applied to the other two gels, which had been treated with a 1 mM solution of potassium clavulanate or 200 µg/ml cloxacillin, respectively. This method allowed on-site distinction between class C  $\beta$ -lactamases, which were sensitive to cloxacillin but insensitive to clavulanate, and class A  $\beta$ -lactamases, which were sensitive to clavulanate but insensitive to cloxacillin (17). *E. coli* clones that harbored TEM-10, TEM-28, SHV-1, and ACT-1 were used as markers for IEF at pI 5.6, 6.1, 7.6, and 9.1, respectively.

**PCR amplification and sequencing.** *Taq* and *Pfu* DNA polymerases were from TaKaRa Biotechnology Co., Ltd. Standard PCR amplification experiments were done with primers specific for the genes coding for  $\beta$ -lactamases OXA-10, TEM, SHV, PER-1, CTX group 1, CTX group 2, CTX group 9, and VEB-1, as described previously (9). The PCR products were purified on Qiaquick columns (QIAGEN) and sequenced on an ABI PRISM 377 automated sequencer (Applied Biosystems, Foster City, Calif.). The nucleotide and deduced amino acid sequences were analyzed with software available over the Internet.

TABLE 1. Sequences of primers for detection of *bla* genes or genotyping of strains

Gene type	Primer	Sequence	Position	Accession no. or reference	Product size (bp)
TEM	TEM-A	5'-ATAAAATTCTTGAAGAC-3'	1-17	X54604.1	1,075
	TEM-B	5'-TTACCAATGCTTAATCA-3'	1075-1059		
SHV	SHV-FOR	5'-TGGTTATGCGTTATATTCGCC-3'	69-89	X98100.1	867
	SHV-REV	5'-GCTTAGCGTTGCCAGTGCT-3'	936-918		
PRE	PER-FOR	5'-AATTTGGGCTTAGGGCAGAA-3'	278-306	Z21957	933
	PER-REV	5'-ATGAATGTCATTATAAAAGC-3'	1211-1192		
VEB	VEB-FOR	5'-CGACTTCCATTTCCCGATGC-3'	226-245	AF220758	642
	VEB-REV	5'-GGACTCTGCAACAAATACGC-3'	868-849		
CTX-M-3	M13U	5'-GGTTAAAAAATCACTGCGTC-3'	65-84	X92506	863
	M13L	5'-TTGGTGACGATTTTAGCCGC-3'	928-909		
CTX-M-9	M9U	5'-ATGGTGACAAAGAGAGTGCA-3'	1-20	AF252621.2	870
	M9L	5'-CCCTTCGGCGATGATCTC-3'	870-852		
CTX-M-2	M25U	5'-ATGATGACTCAGAGCATTGCG-3'	304-323	AJ416343.1	865
	M25L	5'-TGGGTTACGATTTTCGCCGC-3'	1169-1150		
OXA-10	OXA-10-A	5'-GTCTTTTCG(A)AGTACGGCATT-3'	1502-1521	AY115475.1	699
	OXA-10-B	5'-ATTTTCTTAGCGCAACTTAC-3'	2201-222		
REP PCR	REP 2-1	5'-ICGICTTATCIGGCCTAC-3'		21	
	ERIC 2	5'-AAGTAAGTGACTGGGGTGAGCG-3'		18	

TABLE 2. Characteristics of multidrug-resistant *P. aeruginosa* clinical isolates

Strain	$\beta$ -Lactamase pI(s) <sup>a</sup>			PCR result for <sup>b</sup> :								REP PCR type <sup>c</sup>
	No inhibitors	+ Clavulanic acid	+ Cloxacillin	TEM	SHV	OXA-10	CTX-1	CTX-2	CTX-9	VEB	PER	
643	8.2, 7.45, 5.4	8.2	7.45, 5.4	+	—	—	—	—	—	+	—	5
649	7.45		7.45	—	—	—	—	—	—	+	—	5
655	8.2, 7.45	8.2	7.45	—	—	—	—	—	—	+	—	5
660	8.2, 7.45	8.2	7.45	—	—	—	—	—	—	+	—	5
661	8.2, 7.45	8.2	7.45	—	—	—	—	—	—	+	—	5
684	7.45, 6.1, 5.4		7.45, 6.1, 5.4	+	—	+	—	—	—	+	—	1
691	8.2, 7.45	8.2	7.45	—	—	—	—	—	—	+	—	5
701	6.1, 5.4		6.1, 5.4	+	—	+	—	—	—	+	—	1
710	8.2, 7.45	8.2	7.45	—	—	—	—	—	—	+	—	5
721	8.2, 7.45	8.2	7.45	—	—	—	—	—	—	+	—	3
843				—	—	—	—	—	—	—	—	4
848	8.2, 7.45	8.2	7.45	—	—	—	—	—	—	+	—	5
852	7.45, 6.1, 5.4		7.45, 6.1, 5.4	+	—	+	—	—	—	+	—	1
884	8.2, 7.45, 5.4	8.2	7.45, 5.4	+	—	+	—	—	—	+	—	1
891	8.2, 7.45	8.2	7.45	—	—	—	—	—	—	+	—	5
914	6.1, 5.4		6.1, 5.4	+	—	+	—	—	—	—	—	5
918	8.2, 7.45	8.2	7.45	—	—	—	—	—	—	+	—	5
941	7.45, 6.1, 5.4		7.45, 6.1, 5.4	+	—	+	—	—	—	+	—	2
961	7.45, 6.1		7.45, 6.1	—	—	+	—	—	—	+	—	2
964	7.45, 5.4		7.45, 5.4	+	—	—	—	—	—	+	—	2
966	7.45, 6.1, 5.4		7.45, 6.1, 5.4	+	—	+	—	—	—	+	—	1
970	8.2, 7.45	8.2	7.45	—	—	—	—	—	—	+	—	5
974	8.2, 7.45	8.2	7.45	—	—	—	—	—	—	+	—	3
977	6.1, 5.4		6.1, 5.4	+	—	+	—	—	—	—	—	1
991	8.2, 7.45	8.2	7.45	—	—	—	—	—	—	+	—	5
1013	7.45, 6.1, 5.4		7.45, 6.1, 5.4	+	—	+	—	—	—	+	—	1
1038	7.45, 6.1, 5.4		7.45, 6.1, 5.4	+	—	+	—	—	—	+	—	1
1052	8.2, 7.45	8.2	7.45	—	—	—	—	—	—	+	—	1
1073	8.2, 7.45	8.2	7.45	—	—	—	—	—	—	+	—	5
1085	8.2, 7.45	8.2	7.45	—	—	—	—	—	—	+	—	5
1090	8.2, 7.45	8.2	7.45	—	—	—	—	—	—	+	—	5
1092	8.2, 7.45	8.2	7.45	—	—	—	—	—	—	+	—	1
1111	8.2, 7.45	8.2	7.45	—	—	—	—	—	—	+	—	5
1142	6.1, 5.4		6.1, 5.4	+	—	+	—	—	—	—	—	4
1149	7.45, 6.1, 5.4		7.45, 6.1, 5.4	+	—	+	—	—	—	+	—	1
1045	8.2	8.2		—	—	—	—	—	—	—	—	4

<sup>a</sup> Enzymes focused at pI 8.2 that were inhibited by cloxacillin but not inhibited by potassium clavulanate were considered class C enzymes. Enzymes focused at pI 7.45 and 5.4 that were inhibited by potassium clavulanate but not inhibited by cloxacillin were considered class A enzymes. Enzymes focused at pI 6.1 that were not inhibited by potassium clavulanate or cloxacillin were considered class D enzymes.

<sup>b</sup> +, positive; —, negative.

**REP PCR typing.** The multidrug-resistant *P. aeruginosa* isolates were typed by repetitive extragenic palindromic (REP) PCR with primers ERIC 2 (enterobacterial repetitive intergenic consensus sequences) and REP (REP elements) (Table 1) (5, 7). The PCR products were separated in 1.2% agarose gels.

## RESULTS

By disk diffusion susceptibility testing, all 75 of the *Pseudomonas aeruginosa* isolates tested were classified into three main phenotypes according to their susceptibilities to the ESCs, aztreonam, and cefipime. Twenty-nine *P. aeruginosa* isolates were susceptible to all ESCs, aztreonam, cefepime, and imipenem (the ESC-susceptible group). Ten *P. aeruginosa* isolates showed intermediate susceptibility to ceftazidime and were either intermediate or susceptible to cefotaxime, aztreonam, and cefepime (the ESC-intermediate group). Thirty-six *P. aeruginosa* isolates were resistant to all ESC  $\beta$ -lactams (the ESC-resistant group) and cefepime, except one that was susceptible to aztreonam.

The first step in testing was designed to analyze the  $\beta$ -lactamase contents of the isolates. Thirty-five multidrug-resistant

*P. aeruginosa* isolates and 10 ESC-intermediate strains were positive in the nitrocefin test. One ESC-resistant isolate and 29 ESC-susceptible isolates were negative in the nitrocefin test and showed no  $\beta$ -lactamase bands on the IEF gel in the next experiment. IEF, PCR, and PCR product sequences were designed to further characterize the  $\beta$ -lactamase contents. On the IEF gel, one multidrug-resistant isolate and 10 ESC-intermediate *P. aeruginosa* isolates presented a single  $\beta$ -lactamase band (pI range, 7.8 to 9.2), which was inhibited by cloxacillin but not by potassium clavulanate and was thus considered a class C enzyme. Thirty-four ESC-resistant *P. aeruginosa* isolates showed one or more additional  $\beta$ -lactamase bands that focused at pIs of 7.45, 6.1, and 5.4. The enzyme focused at 6.1 was not inhibited by potassium clavulanate or cloxacillin. The enzymes focused at 7.45 and 5.4, which were inhibited by potassium clavulanate but not by cloxacillin, were delete considered class A enzymes.

To obtain more information on the types of ESBLs in the isolates that were positive in the nitrocefin test and that showed class A enzymes in the IEF test, we analyzed the

TABLE 3. Accuracies of various forms of DDST compared to biochemical and genotypic methods<sup>a</sup>

ESBL-screening method	No efflux inhibitor or cloxacillin				Plus efflux inhibitor		Plus cloxacillin		Plus efflux inhibitor and cloxacillin	
	30 mm from AMC		20 mm from AMC		No. positive	Sensitivity (%)	No. positive	Sensitivity (%)	No. positive	Sensitivity (%)
	No. positive	Sensitivity (%) <sup>b</sup>	No. positive	Sensitivity (%)						
CAZ	7	20.6	15	44	26	76.5	26	76.5	30	88.2
CTX	0	0	0	0	0	0	5	14.7	7	20.6
ATM	5	14.7	4	11.8	19	55.9	21	61.8	28	82.4
FEP	10	29.4	23	67.6	29	85.3	25	73.5	31	91.2
At least one combination <sup>c</sup>	10	29.4	25	73.5	29	85.3	26	76.5	32	94.1
CAZ/CLA-CAZ $\geq$ 5 mm	20	58.8			31	91.2	28	82.4	33	97.1
CTX/CLA-CTX $\geq$ 5 mm	1	2.9			3	8.8	8	23.5	11	32.4
At least one combination <sup>d</sup>	20	58.8			31	91.2	29	85.3	33	97.1

<sup>a</sup> The number of ESBL-positive strains was 34. The number of ESBL-negative strains was 41. The results were confirmed by IEF, PCR, and PCR product sequencing. The negative strains included 29 susceptible isolates, 10 intermediate isolates, and 2 multidrug-resistant isolates. No false positive was detected among 41 ESBL-negative isolates.

$$^b \text{Sensitivity} = \frac{\text{real positive}}{\text{real positive} + \text{false negative}}$$

<sup>c</sup> If any one of the CAZ, CTX, ATM, or FEP disks indicated ESBL positive, the DDST result was considered ESBL positive.

<sup>d</sup> If any one of the CAZ/CLA-CAZ, CTX/CLA-CTX tests indicated ESBL positive, the combined disk test result was considered ESBL positive.

$\beta$ -lactamases of these isolates by PCR experiments with a series of primers specific for *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, *bla*<sub>CTX-M-3</sub>, *bla*<sub>CTX-M-9</sub>, *bla*<sub>CTX-M-2</sub>, and *bla*<sub>PER-1</sub>. Three of the seven genes were found alone or in various combinations. *bla*<sub>TEM-1</sub>-like, *bla*<sub>OXA-10</sub>-like, and *bla*<sub>VEB-1</sub>-like genes were found in 13, 12, and 29 of the isolates, respectively. Sequence determination of all of the PCR products showed that the *bla*<sub>TEM-1</sub>-like gene was *bla*<sub>TEM-116</sub>, the *bla*<sub>OXA-10</sub>-like gene was *bla*<sub>OXA-10</sub>, and the *bla*<sub>VEB-1</sub>-like gene was *bla*<sub>VEB-3</sub> (Table 2). Based on the results of IEF and PCR product sequencing, it was determined that pI 6.1 corresponded to OXA-10, pI 7.45 corresponded to VEB-3, pI 5.4 corresponded to TEM-116, and pI 8.2 corresponded to AmpC-type cephalosporinase. The isolates that showed a class C enzyme only in IEF were also analyzed by PCR to confirm the IEF results, but no isolates were positive in any PCR test.

The second step in testing was designed to compare the reliabilities of various ESBL-screening methods for these 75 *P. aeruginosa* clinical isolates. The results are shown in Table 3. Ten isolates were detected as positive by using a conventional DDST with ceftazidime, cefotaxime, aztreonam, and cefepime disks at 30 mm from the central disk of AMC. The number of isolates with positive combined disk test results was 20. When the same disk was applied at a 20-mm distance, the number of isolates with positive DDST results increased to 25. When the DDSTs were performed on cloxacillin (200  $\mu$ g/ml)-containing plates, the number of isolates with positive DDST results increased to 26 and the number with positive combined disk test results increased to 29. When the DDSTs and combined disk tests were performed on efflux pump inhibitor (MC-207,110)-containing plates with the disks at a distance of 20 mm, the number of positive isolates in the DDSTs was 29 and the number of positive isolates in the combined disk test was 31. When the DDSTs (20 mm from AMC) and combined disk tests (30 mm from AMC) were performed on cloxacillin (200  $\mu$ g/ml)- and MC-207,110 (20  $\mu$ g/ml)-containing plates, the number of positive isolates in the DDSTs was 32 and the number of positive isolates in the combined disk tests was 33.

The relationship between the 36 multidrug-resistant *P.*

*aeruginosa* isolates was studied by REP PCR using independent ERIC 2 and REP primers. Both amplifications with ERIC 2 and REP primers gave five distinct types of profiles of the strains. The majority (18 of 20) of *P. aeruginosa* isolates that possessed the enzyme with pI 8.2 belonged to type 5, except *P. aeruginosa* 884 and 974. The majority (11 of 15) of the *P. aeruginosa* isolates without the enzyme with pI 8.2 exhibited the same pattern (REP PCR type I). Both types of *P. aeruginosa* isolates harbored the *bla*<sub>VEB-3</sub> gene. The remaining eight multidrug-resistant *P. aeruginosa* isolates were distributed in three unrelated gene-type patterns (Table 2).

## DISCUSSION

The results of this study showed a high frequency of ESBLs in the samples of *P. aeruginosa* isolates examined. *bla*<sub>VEB-3</sub>, which was first detected in *Enterobacter cloacae* and now appeared in *P. aeruginosa* for the first time, was the most frequently encountered ESBL gene in our study (30 of 75) (9). Based on REP PCR typing, the VEB-3 ESBL-producing isolates seemed to have no relationship with REP gene types. The presence of the same  $\beta$ -lactamase in different types of strains indicated that horizontal gene spread might be responsible for the high frequency of ESBLs detected in *P. aeruginosa* in this study. Another ESBL found in these *P. aeruginosa* clinical isolates was TEM-116. These observations suggest that ESBLs widespread in the family *Enterobacteriaceae* might be increasingly frequently found in *P. aeruginosa*, which could also be a reservoir for the dissemination of this kind of enzyme (11).

Current ESBL detection methods for *P. aeruginosa* are unreliable, and the reported numbers of ESBL producers are generally low because of mutational derepression of the chromosomally mediated AmpC enzyme, up regulation of efflux systems, and decreased outer membrane permeability (11). With the occurrence of ESBLs in *P. aeruginosa* being increasingly reported worldwide (2, 12, 14, 15, 20, 21), there is a need for a reliable test to detect ESBLs in clinical isolates of *P. aeruginosa*. The test also needs to be practical for routine use in the clinical laboratory.

In a recent study, a DDST method that combined AMC with cefepime successfully detected the SHV-5  $\beta$ -lactamase in a *K. pneumoniae* strain that produced a plasmid-borne AmpC enzyme (9a). In another report, the use of cefepime increased the sensitivity of the DDST with ESCs for the detection of ESBLs in enterobacters from 16% to 61% when the disks were applied at the standard distance of 30 mm from AMC and from 71% to 90% with a shorter distance (20 mm) for application of the disks (19). These results suggested that the inhibition of the activities of the AmpC enzyme and efflux pumps might enhance the abilities of DDSTs to detect ESBLs in *P. aeruginosa*.

The results of our work support previous suggestions that the current ESBL detection methods, which are based on the inhibitory effect of clavulanic acid on the activities of ESBLs against ESCs, are inadequate in cases of overproduction of *P. aeruginosa* (12, 19). The conventional DDST with disks applied at a distance of 30 mm from AMC was the least sensitive method in our setting. When the disks were applied at the standard distance of 30 mm from AMC, the use of cefepime increased the sensitivity of the DDST with ESCs for the detection of ESBLs in *P. aeruginosa* from 20.6% to 44%. When the disks were applied at a shorter distance of 20 mm from AMC, the use of cefepime increased the sensitivity of the tests from 29.4% to 67.6%. The results showed that the distance between disks is also an important factor for the detection of ESBLs in *P. aeruginosa* by the DDST. When the DDST and combined disk test were performed on both MC-207,110 base(20  $\mu$ g/ml)- and cloxacillin (200  $\mu$ g/ml)-containing plates, the sensitivity increased to 91.2% and 97.1%, respectively. The results showed that inhibiting the activities of the AmpC enzyme and multidrug efflux could increase the sensitivity of the DDST for detection of ESBLs in *P. aeruginosa*. However, further research on the subject is beyond the range of this study.

In our study, the combined disk test with CAZ-CAZ/CLA was a more efficient test than that with CTX-CTX/CLA for detection of ESBLs in *P. aeruginosa*. The combined disk tests with CAZ-CAZ/CLA and CTX-CTX/CLA were positive for 20 isolates and 1 isolate, respectively, when the tests were performed on MH plates. The combined disk tests performed on both MC-207,110 (20  $\mu$ g/ml)- and cloxacillin (200  $\mu$ g/ml)-containing plates had the best sensitivity in our work. Only one strain was falsely negative because the enhancement zone was too big to be visualized on the plate. No ESBLs or AmpC enzyme was detected in *P. aeruginosa* isolate 843. This result occurred not only in IEF and ESBL PCR, but also in the DDST and combined disk test performed on both MC-207,110 (20  $\mu$ g/ml)- and cloxacillin (200  $\mu$ g/ml)-containing plates. The consistency of the results of IEF, ESBL PCR, and ESBL screening tests shows a high reliability for the DDST and combined disk test in detecting ESBLs in multidrug-resistant *P. aeruginosa* when the activities of the AmpC enzyme and efflux pumps are inhibited. No ESBL false positive was detected in any of the the ESBL-screening methods for those isolates which were ESBL-negative in IEF, PCR, and PCR product sequencing (Table 3).

In general, ESBLs resulted in very high levels of ESC resistance, whereas hyperproduction of AmpC and/or impermeability/efflux function gave lower levels resistance (12). The high frequency of ESBLs in our work indicates the necessity for monitoring ESBL-producing strains of *P. aeruginosa*, and

the DDST and combined disk test performed on MC-207,110 (20  $\mu$ g/ml)- and cloxacillin (200  $\mu$ g/ml)-containing plates were efficacious tests for the detection of ESBLs in *P. aeruginosa*.

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